

Drugs. GA and 17AAG were obtained from the Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis of the National Cancer Institute in the form of a lyophilized powder. They were stored in dark, airtight containers at 4°C and reconstituted in DMSO immediately before use.

Clonogenic Assays. Cytotoxicity of GA and 17AAG was determined by clonogenic assay. Four $\times 10^2$ C6 cells or 5×10^2 T98G cells were plated in 60-mm tissue culture dishes and allowed to adhere overnight. The cells were then exposed to various concentrations of drug ranging from 0.05–100 nM (control cultures were treated with 0.01% DMSO) and incubated for 24–48 h at 37°C. The medium was then removed and replaced with fresh medium free of drug. After 7–10 days of growth, colonies were fixed and stained with 0.5% methylene blue. Colonies with diameters of 2 mm and larger were counted using an electronic counting pencil.

Preparation of Cell Homogenates for Detection of Cytosolic EF-2 Kinase. Cell monolayers were washed twice in PBS (pH 7.4), scraped into 15-ml centrifuge tubes in 5 ml of PBS, and then centrifuged at $1,000 \times g$ for 5 min. Cell pellets were homogenized with 15 strokes in ice-cold HOMO buffer [25 mM HEPES (pH 7.4), 100 mM sodium chloride, 20 mM sodium PPi, 2 mM EDTA, 0.1 mM PMSF, 10 μ g/ml leupeptin, 2 μ g/ml pepstatin A, and 0.1 mM sodium orthovanadate], using a Polytron homogenizer. The homogenates were then centrifuged at $15,000 \times g$ for 30 min at 4°C. The protein concentration of the supernatants was determined according to the method of Bradford using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA). Twenty μ g of protein from each sample were used for EF-2 kinase detection. Sample volumes were adjusted with HOMO buffer. Samples were boiled with 3X Laemmli buffer [190 mM Tris (pH 6.8), 6% SDS, 30% glycerol, 15% 2-mercaptoethanol, and 0.003% bromphenol blue dye] for 5 min before resolution by 7.5% SDS-PAGE.

Immunoprecipitations. Cell monolayers were washed twice with PBS and lysed with NET lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% NP40, 1.0 mM EDTA, 1 mM PMSF, and 1% aprotinin] at 4°C for 30 min. Cells were scraped into 1.5-ml microcentrifuge tubes and incubated at 4°C with rotation for 30 min, passed through a 25-gauge needle, and then centrifuged at $15,000 \times g$ at 4°C for 30 min. The protein concentration of the supernatants was determined using the Bio-Rad protein assay kit.

Immunoprecipitations. Immunoprecipitations were performed using 2 mg (C6 cells) or 1 mg (T98G cells) of protein from total cell lysates as starting material. Reaction volumes were equalized using NET buffer. For Hsp90 precipitation, 4 μ g of anti-Hsp90 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were added to each sample. For EF-2 kinase precipitation, 4 μ l of anti-EF-2 kinase rabbit serum (kindly provided by Dr. A. C. Nairn of Rockefeller University, New York, NY) was used. Immunoprecipitation of samples with preimmune sera was used as a control. After 1–4 h of rotating incubation at 4°C, 80 μ l of Protein A-Sepharose CL-4G (50% NET buffer; Amersham Pharmacia Biotech, Piscataway, NJ) were added. Tubes were incubated overnight at 4°C with rotation. At the end of the incubation, samples were centrifuged at $15,000 \times g$ at 4°C for 2 min, and the supernatant was discarded. Beads were washed twice with NET buffer and twice with PBS containing 1% aprotinin and 1 mM PMSF. After the final wash, 30 μ l of Laemmli buffer were added, and the samples were boiled for 5 min. After a 3-min spin at $15,000 \times g$, supernatants were loaded onto a 8% SDS-PAGE gel.

Western Blot Analysis. Proteins resolved on SDS-PAGE were transferred to nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech). Membranes were first blocked with 10% milk in PBS-T (PBS/0.1% Tween) and then were incubated with anti-EF-2 kinase rabbit antiserum in 10% milk/PBS-T for EF-2 kinase detection. For detection of Hsp90, the anti-Hsp90 rabbit antiserum was used. An anti- β -actin monoclonal mouse ascites (Sigma Chemical Co., St. Louis, MO) was used for detection of β -actin. After a 2-h incubation with the primary antibodies, membranes were washed in PBS-T, incubated with horseradish peroxidase conjugated secondary antibodies (Amersham-Life Science), and washed again with PBS-T. M_r standards (Bio-Rad Laboratories, Richmond, CA) and authentic EF-2 kinase were used to determine the position of the proteins of interest. Proteins were visualized using Amersham-Pharmacia ECL detection reagents. Blots were scanned by UMAX Magic Scan 4.3 program, and the intensity of protein bands was quantified by Molecular Analyst software.

Generation of T98G Cell Lines Expressing Different Levels of EF-2 Kinase. pSTAR vector was originally constructed to measure tetracycline-induced gene expression in mammalian cells (28). However, this vector conferred constitutive expression of target genes in most stable clones that we have tested, which was likely attributable to the use of two strong promoters, cytomegalovirus and SV40, in this vector system. A full-length human EF-2 kinase cDNA was inserted into the multiple cloning site region of pSTAR in both antisense (AS) and sense (S) orientations. Constructs expressing AS (pTY1) and sense (pTY2) EF-2 kinase mRNA were introduced into T98G human glioma cells by liposome-mediated transfection (Life Technologies, Inc., Rockville, MD). Transfectants were selected in 500 μ g/ml G418. G418-resistant colonies were expanded and maintained in 200 μ g/ml G418. Cytosolic extracts of each clone were collected by homogenization and centrifugation and were analyzed for EF-2 kinase expression by Western blot. The T98G-R clone was found to express low amounts of EF-2 kinase protein. The T98G-D clone expressed high levels of sense EF-2 kinase mRNA and protein compared with that of T98G-R.

Treatment of Glioma Xenografts with 17-AAG. C6 cells grown at logarithmic phase were removed from culture flasks by trypsinization, then washed and resuspended in PBS at density of 1.6×10^7 cells/ml. Cells (1.6×10^6 /100 μ l) were injected into the flank of each of 16 Swiss nude (^{nude}) mice (male, 5 weeks old; Taconic, Germantown, NY). The s.c. grown tumors were measured daily with a Vernier caliper. The length (L) and width (W; in mm) of the tumors were taken and used to calculate the volume (V), using the formula: $V = (W^2 \times L)/2$. On the 12th day after inoculation, when the median tumor volume reached 60 mm³, the mice were paired by the similarity of the tumor volumes. Within each pair, mice were randomly assigned to either 17-AAG or vehicle. Animals received i.p. injections of 17-AAG (80 mg/kg) or vehicle on days 2, 3, 4, 5, and 8, 9, 10, 11, and 12.

Statistical Analysis. The two-sample *t* test was used to compare tumor volumes between the two treatment groups at treatment day 1. Log(tumor volume) on day 5 to day 22 were analyzed using the repeated-measurement model with treatment and day as factors and the first order autoregressive correlation structure (29).

RESULTS

Effect of GA on Cell Growth and Viability. We investigated the effects of a 48-h incubation with GA and 17-AAG on the clonogenicity of human T98G (glioblastoma), Daoy (medulloblastoma) and SKNSH (neuroblastoma) cells. GA inhibited T98G, Daoy, and SKNSH cell viability with IC_{50} values of 3 nM, 1 nM, and 1 nM, respectively (Fig. 1A; Table 1). GA also inhibited the clonogenicity of the C6 rat glioma line (IC_{50} , 1.5 nM).

17-AAG also decreased the clonogenicity of glioma cells, but was ~10-fold less potent than GA against the same cell lines. IC_{50} value for C6 cells was 13 nM and for T98G cells was 35 nM (Fig. 1B; Table 1).

For comparison, we also performed clonogenic assays of C6 cells treated with BCNU, a standard chemotherapeutic drug used to treat glioblastoma. Fig. 1C and Table 1 show that the IC_{50} of BCNU against C6 cells was 5 μ M, ~3000-fold greater than the IC_{50} value of GA against the same cell line.

Effect of GA and 17-AAG on EF-2 Kinase Protein Content. To analyze the role of EF-2 kinase in the cytotoxicity of GAs, we tested whether or not GA or 17-AAG affected EF-2 kinase content in glioma cells. T98G cells were incubated with 0–40 nM GA for 24 h (Fig. 2A, left panel). Cell homogenates were collected and EF-2 kinase measured by Western blot. Fig. 2 demonstrates that there was a >50% decrease in EF-2 kinase at a concentration of 10 nM and a >80% decrease after treatment with 20 nM GA. We chose the 24-h incubation time to avoid problems in interpretation of data obtained using a dying population of cells (Fig. 1). Similar results were observed in C6 cells treated with GA for 24 h (Fig. 2B); EF-2 kinase was reduced by 70% after treatment with 1–10 nM GA. 17-AAG (20–40 nM) decreased EF-2 kinase by 40–50% (Fig. 2C, right panel).

EFFECTS OF GA ON GLIOBLASTOMA AND EF-2 KINASE

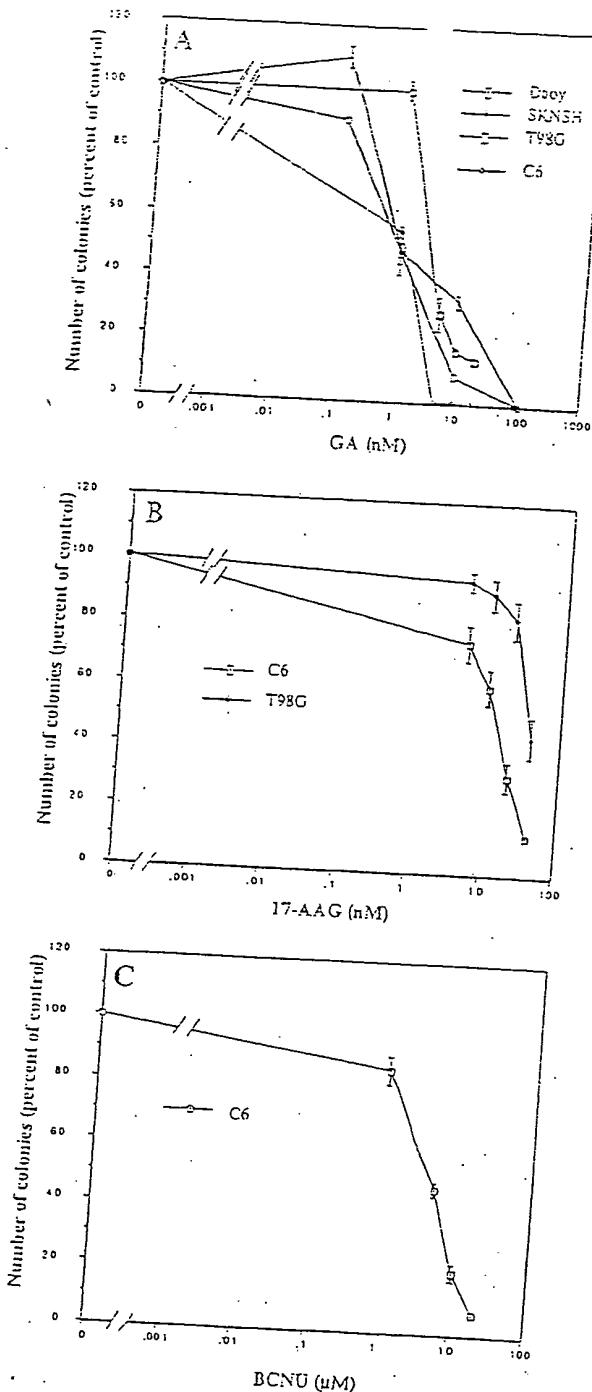


Fig. 1. Effect of GA, 17-AAG, and BCNU on the clonogenic survival of glioma and other cell lines of neurogenic origin. Murine glioma (C6), human glioblastoma (T98G), and human neuroblastoma (SKNSH), and human medulloblastoma (Daoy) were cultured as described in "Materials and Methods." Cell lines were exposed for 48 h in varying concentrations of GA (A), 17-AAG (B), and BCNU (C). Cells were then cultured in drug-free medium and allowed to grow for another 7–10 days. Colonies were stained with methylene blue and counted with an electron counting pencil. Each point represents the mean of three determinations from one of three similar experiments; bars, \pm SE. Controls (vehicle-treated cells) usually contained 100–300 colonies.

Effect of GA and 17-AAG on Hsp90/EF-2 Kinase Interactions. We next carried out coimmunoprecipitation experiments to investigate the effects of GA and 17-AAG on Hsp90/EF-2 kinase protein interactions. T98G (Fig. 3A) and C6 cells (Fig. 3B) were treated with 10 nM GA, 40 nM 17-AAG, or vehicle (0.1% DMSO) for 24 h (the optimal conditions for reducing the cellular content of EF-2 kinase as shown above). Cell lysates were incubated with anti-Hsp90 rabbit

antiserum or preimmune serum and protein-A beads to immunoprecipitate Hsp90. After Western transfer, the membranes were first stripped and reblotted with anti-EF-2 kinase polyclonal antibody and then were analyzed with anti-Hsp90 polyclonal antibody. Fig. 3A and B, demonstrate that approximately equal amounts of Hsp90 (bottom panels) was coprecipitated under each condition. EF-2 kinase (Lane 1) but not with preimmune serum (data not shown), which confirmed the association of the kinase with Hsp90 in both T98G and C6 cells. Treatment with GA (Lane 2) or 17-AAG (Lane 3) significantly reduced the amount of EF-2 kinase that was coprecipitated with Hsp90 in both cell lines.

To exclude the possibility that the apparent decrease of Hsp90 association with EF-2 kinase was merely the result of decreased overall EF-2 kinase protein, we investigated the effect of shorter incubation times with higher concentrations of drug. In these experiments, cells were incubated with 3 μ M GA (Lane 4) or 6 μ M 17-AAG (Lane 5) for 3 h. Fig. 3C demonstrates that the amount of EF-2 kinase in T98G or C6 cells was not significantly changed under these conditions. The amount of EF-2 kinase that was coimmunoprecipitated with Hsp90 under these conditions was markedly decreased (Fig. 3A and B, Lanes 4 and 5).

We next determined whether antibodies to EF-2 kinase could immunoprecipitate Hsp90 and whether GA disrupted the interaction. Immunoprecipitation was performed using an anti-EF-2 kinase anti-

Table 1 Effect of GAs on the clonogenicity of glioma and other cell lines of neurogenic origin

Cell lines, grown in tissue culture, were treated with varying concentrations of drugs or vehicles, and clonogenic survival was measured as described in "Materials and Methods." Each value represents the mean of at least two experiments having less than 10% variation between them.

Cell line	GA IC_{50} (nM)	17-AAG IC_{50} (nM)	BCNU IC_{50}
C6	2 ± 0.1		
T98G	3 ± 0.2	13 ± 1.8	5 ± 0.2
SKNSH	1 ± 0.1	35 ± 4.0	
Daoy	1 ± 0.1		
TJY1-R	0.4 ± 0.04		
TJY2-D	2 ± 0.1		

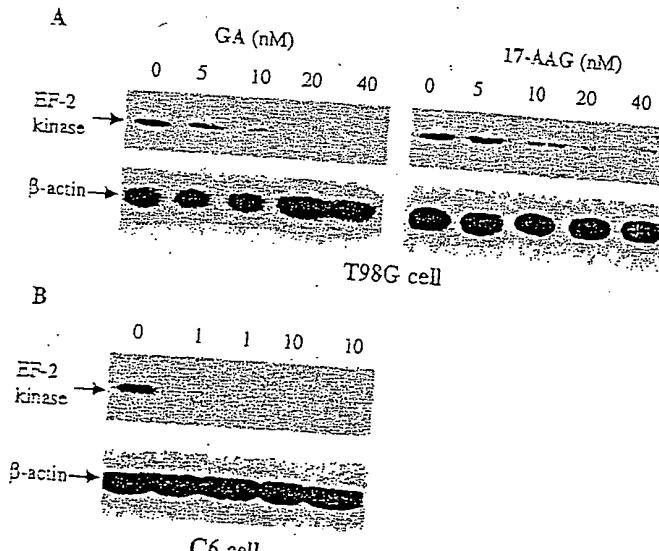


Fig. 2. Effect of GAs on the content of EF-2 kinase in glioblastoma cell lines. T98G (A) and C6 cells (B) were grown in culture as described in "Materials and Methods." Following a 24-h incubation with drug or appropriate vehicle, cell homogenates containing 20 μ g of total protein were assayed for the content of elongation factor 2 kinase and β -actin by Western blot analysis. The figure represents the results of one of three similar experiments.

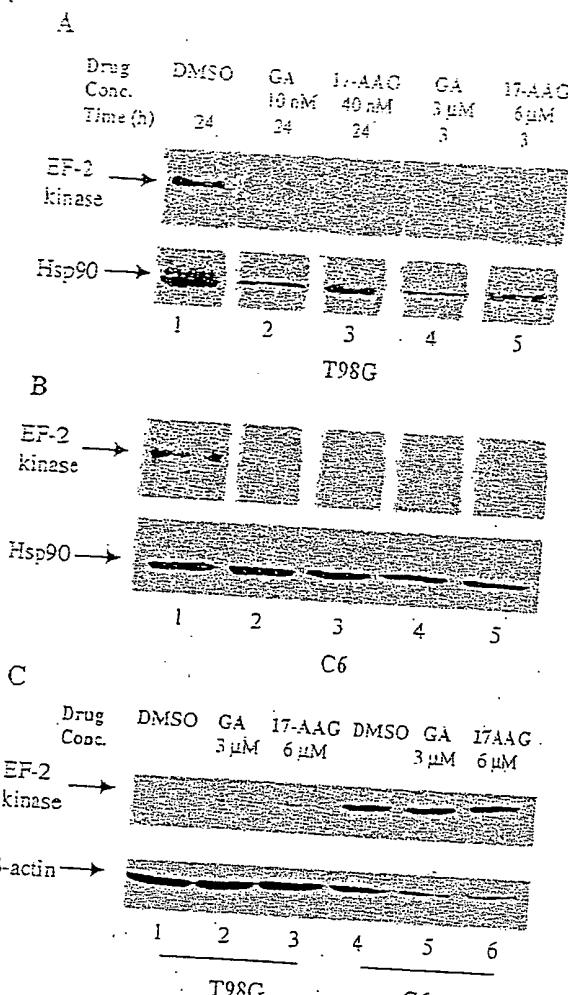


Fig. 3. Immunoprecipitation of EF-2 kinase by antibodies against Hsp90 in cell lysates treated with GAs. T98G (A) and C6 (B) cells were treated with 10 nM GA (Lane 2), 40 nM 17-AAG (Lane 3), or vehicle (0.1% DMSO, Lane 1) for 24 h. Cells were also treated with 3 μ M GA (Lane 4) or 6 μ M 17-AAG (Lane 5) for 3 h. Cell lysates were incubated with anti-Hsp90 rabbit antiserum and protein-A beads to immunoprecipitate Hsp90. After Western transfer, the membranes were first probed with anti-EF-2 kinase polyclonal antibody, then were stripped and reprobed with anti-Hsp90 polyclonal antibody. C. Western blot analysis of EF-2 kinase in T98G and C6 cells treated with GA or 17-AAG in high concentration and shorter incubation time. Cells were incubated with vehicle (Lane 1 and 4), 3 μ M GA (Lanes 2 and 5), or 6 μ M 17-AAG (Lanes 3 and 6) for 3 h. Each blot is representative of three similar experiments.

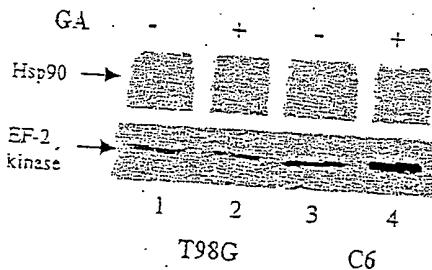


Fig. 4. Immunoprecipitation of Hsp90 by antibodies against EF-2 kinase in cell lysates treated with GAs. T98G and C6 cells were treated with either 3 μ M GA or vehicle (0.1% DMSO) for 3 h. Cell lysates were incubated with anti-EF-2 kinase rabbit antiserum and protein-A beads as described in "Materials and Methods." After Western transfer, the membrane was first probed with anti-Hsp90 polyclonal antibody, then was stripped and reprobed with anti-EF-2 kinase polyclonal antibody. These data are representative of two similar experiments.

body and cell lysates from T98G and C6 were treated with either vehicle or 3 μ M GA for 3 h (Fig. 4). Similar amounts of EF-2 kinase were recovered under each condition (lower panel). Hsp90 was co-precipitated with EF-2 kinase in vehicle controls (Lanes 1 and 3) but

was absent in the immune-complex from GA-treated cell lysates (Lanes 2 and 4).

Effect of Overexpression of EF-2 Kinase on GA Activity. We next determined whether the effect of GA on cytotoxicity could be abrogated by overexpressing EF-2 kinase. In these experiments, we compared the sensitivity to GA in two cell lines that express different levels of EF-2 kinase. T98G and T98D cell lines were isolated after stable transfection of T98G cells with either antisense or sense EF-2 kinase expression vectors. T98D was selected based on low kinase expression after transfection with antisense RNA; T98D was selected as a clone expressing high EF-2 kinase after transfection with sense RNA. The Western blot in Fig. 5A shows that T98D cells express ~6-fold more EF-2 kinase protein as compared with that of T98I-R cells.

Clonogenic assays were performed on T98I-R and T98D cells treated with different concentrations of GA for 24 h. Overall, we noted a decreased viability of clones transfected with EF-2 kinase antisense. Of the several clones identified, T98I-R was the most robust based on morphology and a doubling time that was consistent with the sense transfectants (data not shown). Fig. 5B and Table 1 demonstrate that cells that overexpress EF-2 kinase were 5-fold less sensitive to the cytotoxic effects of GA (IC_{50} , 2 nM) than isogenic cells with less EF-2 kinase content (IC_{50} , 0.4 nM).

Effect of 17-AAG on Glioma Growth in Mice. To test whether GAs remained active against glioma cell lines *in vivo*, we evaluated the effect of 17-AAG on the growth of C6 glioma cells in nude mice. C6 cells were implanted in sixteen mice in the dorsal flank; tumors were allowed to form for 12 days and reached 60 mm³ in size before

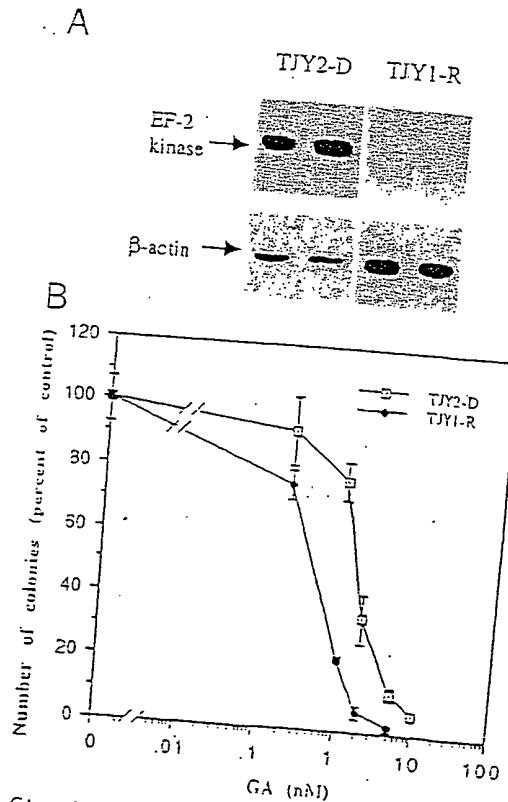


Fig. 5. GA sensitivity of T98G cell derivatives expressing different levels of EF-2 kinase. A, expression of EF-2 kinase in T98 cells transfected with EF-2 kinase sense (T98D) or antisense (T98I-R) expression vectors. EF-2 kinase and β -actin were detected by Western blot analysis. B, effect of GA on the clonogenic survival of T98D and T98I-R cell lines. Drug sensitivity was determined by exposing cells to 0–100 nM GA for 24 h, by washing and allowing the cells to grow for another 7–10 days, and by staining with methylene blue. Colonies were then enumerated using an electronic counting pencil as described in "Materials and Methods." Each point represents the mean from a representative of two similar experiments; bars, \pm SE.

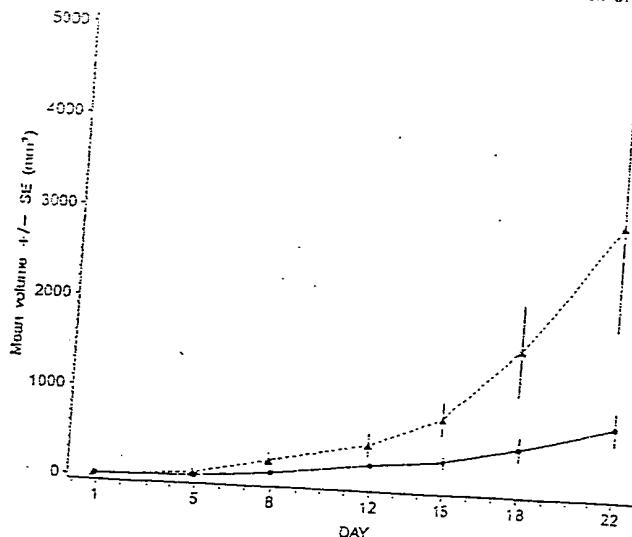


Fig. 6. Effect of 17-AAG on the growth of C6 glioma xenografts. C6 cells (1.6×10^6 cells/mouse) were implanted into the flank of 16 Swiss nude (^{nu/nu}) mice. On the 12th day after inoculation (day 1 in Fig. 6), the mice were paired in two groups and were randomly assigned to treatment. 17-AAG (80 mg/kg) or vehicle were injected via the intraperitoneal route on days 2, 3, 4, 5, 8, 9, 10, 11, and 12. Each point represents the mean volume of the eight animals in each group; bars, \pm SE. The differences in tumor volumes between the two treatment groups were statistically significant ($P \leq 0.017$) at all time points from day 5 to day 22 [least-square mean of $\log(\text{tumor volume})$, 0.91 ± 0.33 (mean \pm SE)].

treatment began. Fig. 6 demonstrates the ability of 17-AAG to significantly inhibit the growth of C6 glioma. The mean tumor volumes of the two treatment groups were not statistically different before drug treatment (day 1; $P > 0.752$). The differences in tumor volumes between the two treatment groups were statistically significant ($P \leq 0.017$) at all time points from day 5 to day 22 [least-square means of $\log(\text{tumor volume})$, 0.91 ± 0.33 (mean \pm SE)]. No significant weight loss, changes in activity or deaths were observed during the period of drug treatment.

DISCUSSION

The need for new treatments for glioblastoma is highlighted by its devastation to both children and adults. Despite the recent introduction of an oral alkylating agent, temozolomide (30–32), very little has been added to prospects for increasing overall survival in patients with this disease.

Disruption of Hsp90 chaperoning represents a novel approach to developing new anticancer therapies. Numerous proteins induced in the growth of malignant cells associate with Hsp90 (22, 33). Interference with this interaction targets the nonassociated protein for degradation (34–38). Therefore, it may be possible to target proteins important for the growth of malignant cells by disrupting their association with Hsp90.

EF-2 kinase is an attractive target for the treatment of high-grade astrocytomas and perhaps other malignancies. The markedly increased activity of this enzyme in glioblastoma multiforme (17) and the cytotoxicity of specific and nonspecific inhibitors for this kinase support this concept (18). Because EF-2 kinase is chaperoned by Hsp90 (20, 21), we examined the effects of GAs on glioma and other malignant cell lines of neurogenic origin and found these drugs to be potent cytotoxic agents that disrupt Hsp90/EF-2 kinase interactions.

GA is a potent inhibitor of the clonogenicity of several cell lines including T98G and C6 glioblastoma, Daoy medulloblastoma, and SKNSH neuroblastoma (Fig. 1). Table 1 lists the IC_{50} values obtained after a 48-h exposure to GA that ranged from 1 to 3 nm. For C6 cells, GA is 2500 times more potent than BCNU, a standard chemotherapy

for glioblastoma (39–42). 17-AAG, a less potent inhibitor of Hsp90/protein interactions (25, 26) compared with GA, is also a less potent inhibitor of clonogenicity in T98G and C6 cells, with IC_{50} values ranging from 13 to 35 nm (Fig. 1; Table 1). It is nevertheless a potent compound when compared with BCNU.

To determine whether or not the cytotoxicity of GAs was attributable, at least in part, to inhibition of EF-2 kinase activity through disruption of Hsp90 chaperoning, we carried out a series of experiments to measure both the association and depletion of the enzyme. These studies revealed a good correlation between the concentration of drug required to disrupt Hsp90/EF-2 kinase interactions and effects on clonogenic survival. For example, after a 24 h incubation of T98G cells with 10 nm GA there is a >50% decrease in EF-2 kinase (Fig. 2A), which correlates with the IC_{50} of 3 nm obtained by clonogenic assay after exposing the cells to drug for 48 h (Fig. 1; Table 1). We chose the shorter incubation period to evaluate the effect of drug exposure on kinase degradation to avoid the pitfalls of studying the effects of drug in a population in which one-half the cells were dead or dying. Similarly, we found a 70% decrease in EF-2 kinase in C6 cells exposed for 24 h to 10 nm GA, which correlates with the IC_{50} value of 2 nm obtained by clonogenic assay after exposing the cells to drug for 48 h. The IC_{50} s in clonogenic assays for 17-AAG were 5- to 10-fold greater than those of GA, which correlates to the decreased potency of this derivative in producing loss of the kinase (Fig. 2A).

Interference with the chaperoning function of Hsp90 is known to be detrimental to associated kinases. GA disrupts the binding of EF-2 kinase to Hsp90 as measured by coimmunoprecipitation (Fig. 3 and 4). The disruption of this interaction is associated with decreased EF-2 kinase protein content (Fig. 2). Schulte *et al.*, demonstrated that the serine/threonine kinase, raf-1, is rapidly destabilized on addition of GA, which disrupts the Raf-1/Hsp90 molecular complex (38). The disappearance of EF-2 kinase in glioma cells treated with GA is likely caused by this mechanism. These data are consistent with a proposed mechanism by which GAs disrupt the chaperoning of critical cellular proteins leading to targeted degradation (38, 43, 44). Further insight may be gained by investigating the mechanism by which the EF-2 kinase protein is degraded. Published reports (45–47) point toward the role of GA in targeting proteins for proteasome degradation.

GAs are likely to affect numerous intracellular targets that are critical for cell viability. In addition to raf-1 (26, 48–50), both GA and 17-AAG deplete cells of the transmembrane protein tyrosine kinase p185^{erb-2}, which has been implicated in oncogenic transformation (45, 51). Treatment of lung cancer cell lines expressing p185 with paclitaxel and 17-AAG showed synergistic activity (52). 17-AAG also destabilizes mutant forms of p53 (47). Therefore, to understand the importance of EF-2 kinase among these other targets, we transfected parental T98G cells with either sense or antisense EF-2 kinase expression vectors. As expected, overexpression of antisense mRNA and subsequent decrease in EF-2 kinase (Fig. 5A) reduced the viability and subsequent recovery of transfected clones (53). Nonetheless, we were able to study drug sensitivity by choosing our most robust antisense clone for these experiments and compared this with a sense clone obtained during the same round of transfections. We then chose a shorter drug exposure time (24 h) to measure effects on clonogenicity and repeatedly found the antisense clone to be more sensitive to GA (Fig. 5B). This result is consistent with EF-2 kinase being an important target for the cytotoxicity of GAs in glioma cell lines. However, despite the excellent correlation between EF-2-kinase content and drug sensitivity (Fig. 5, A and B), given the difficulty in recovering viable antisense clones, we cannot rule out the

possibility that these are indirect effects resulting from clonal selection.

Numerous drugs kill cancer cells in tissue culture but far fewer retain activity in animal xenografts. We studied 17-AAG against xenografted C6 glioma because it is a less toxic compound that has already entered clinical testing (26,27). 17-AAG significantly inhibits the growth of established gliomas in animals. Treatment that is started after 12 days of growth significantly inhibits the growth of the xenografts (Fig. 6). This is particularly promising because the drug is highly lipid-soluble and likely to penetrate the blood-brain barrier.

In summary, our results demonstrate that GAs represent a new class of anticancer drug that have potent activity against glioblastoma and other malignant cell lines of neurogenic origin. The compounds disrupt the interaction of EF-2 kinase with Hsp90, leading to the destruction of this unique protein kinase member of the calmodulin-mediated pathway of signal transduction.

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